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ABSTRACT

The prothrombotic mediator thromboxane A_2 is derived from arachidonic acid metabolism through the cyclooxygenase and thromboxane synthase pathways, and transduces its effect through the thromboxane prostanoid (TP) receptor. The aim of this study was to determine the effect of the TP receptor antagonist and thromboxane synthase inhibitor EV-077 on inflammatory markers in human umbilical vein endothelial cells and on human coronary artery smooth muscle cell proliferation. To this end, mRNA levels of different proinflammatory mediators were studied by real time quantitative PCR, supernatants were analyzed by enzyme immune assay, and cell proliferation was assessed using WST-1. EV-077 significantly decreased mRNA levels of ICAM-1 and PTX3 after TNFα incubation, whereas concentrations of 6-keto PGF1α in supernatants of endothelial cells incubated with TNFα were significantly increased after EV-077 treatment. Although U46619 did not alter coronary artery smooth muscle cell proliferation, this thromboxane mimetic enhanced the proliferation induced by serum, insulin and growth factors, which was significantly inhibited by EV-077. In conclusion, EV-077 inhibited TNFα-induced endothelial inflammation and reduced the enhancement of smooth muscle cell proliferation induced by a thromboxane mimetic, supporting that the thromboxane pathway may be associated with early atherosclerosis in terms of endothelial dysfunction and vascular hypertrophy.

1. Introduction

Thromboxane A_2 (TXA_2), a member of the prostaglandin cascade, is a potent prothrombotic mediator with profound effects on vascular reactivity and platelet activation. This lipid mediator is synthesized from arachidonic acid through the cyclooxygenase (COX) pathway and a specific thromboxane synthase (TXAS). In target cells, such as platelets, endothelial cells (ECs) and vascular smooth muscle cells (SMCs), the biological responses of TXA_2 are transduced through the G-protein coupled cell membrane thromboxane receptor, denoted TP. Targeting TXA_2 through the common low-dose aspirin treatment for prevention of thrombosis relies on the irreversible inhibition of COX-1 in platelets, which lack the ability to resynthesize COX enzymes, leading to a selective inhibition of platelet TXA_2 formation.

Thromboxane is released during vascular inflammation, such as atherosclerosis [1] and vascular injury [2]. Interestingly, analysis of human atherosclerotic lesions revealed that TXAS was expressed in infiltrating macrophages, suggesting immune cells as a potentially important source of TXA_2, in addition to platelets [1]. The latter notion has been supported by the association of the urinary thromboxane metabolite 11-dehydro TXB_2 with systemic inflammation [3]. Taken together, these findings open up for a role of thromboxane in atherosclerosis and vascular injury, which goes beyond platelet aggregation. In support of the latter notion, either genetic or pharmacological targeting of the TP receptor reduces atherosclerosis in hyperlipemic mice [4–9], an effect which is not observed with aspirin treatment [10].

Thromboxane A_2 and isoprostanes can stimulate endothelial TP receptors and increase the expression of proinflammatory markers such as ICAM-1 and VCAM-1 [6]. In animal models, antagonism of the TP receptor preserves endothelium-dependent relaxation of

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isolated vessels [11], and clinical trials have shown improved endothelial function in patients with coronary artery disease by addition of the TP receptor antagonist terutroban to aspirin [12].

Whereas the vasoconstrictive effect of TP receptor signaling is well established [13,14], only few studies have addressed the direct effects of thromboxane mimetics on human vascular SMCs. In aortic subendothelial intimal cells isolated from autopsy material by enzymatic digestion of atherosclerotic plaques, the stable TXA_2 analogue U46619 stimulated [^{3}H]thymidine uptake [15], suggesting that TP receptor signaling may transduce SMC proliferation. However, animal studies have generated conflicting results about the role of TXA_2 signaling in vascular SMC growth. In an initial study, 9,11-epithio-11,12-methano-TXA_2 (STA_2) stimulated DNA synthesis in rat vascular SMCs derived from normotensive WKY rats, but not in those derived from spontaneously hypertensive rats (SHR) [16]. Whereas the stimulation of SMC proliferation was confirmed by subsequent studies of different thromboxane mimetics, such as U46619, STA_2 or carbocyclic TXA_2 (CTA_2) [17–19], other investigators reported that U46619 did not alter rat aortic SMC proliferation [20,21]. Nevertheless, the response to U46619 may be dose-dependent [22] and may vary whether or not experiments were performed in the presence of other growth stimuli.

EV-077 is a reversible dual TP receptor antagonist and thromboxane synthase inhibitor, which inhibits platelet aggregation both *in vitro* in response to either arachidonic acid or U46619 [23–25] and after oral administration [26]. However, the effects of EV-077 on human endothelial and vascular smooth muscle cells have not previously been examined. Based on the above cited findings, the aim of the present study was to determine the effects of EV-077 on inflammatory markers in human umbilical vein endothelial cells (HUVEC) and on human coronary artery SMC proliferation.

2. Materials and methods

2.1. Materials

The TXA_2 mimetic U46619 [(15*S*)-hydroxy-11α,9α-(epoxymethano)-presta-5*Z*,13*E*-dienoic acid] was purchased from Cayman Chem (Ann Arbor). TNFα was purchase from R&D Systems. The reversible dual TP receptor antagonist and thromboxane synthase inhibitor EV-077 was obtained from Evolva (Reinach, Switzerland).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Switzerland). HUVEC were cultured in endothelial cell growth medium, EGM-2 (Lonza), and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were used at passages 2–4. Human coronary artery SMC was purchased from Clonetics (Cambrex Bio Science, Walkersville, MD), and cultured in SmGM2 kit medium and used at passages 5–8 as previously described [27].

2.3. HUVEC incubation

HUVEC were seeded at 67,500 cells/flask in either gelatin-coated T25 in EGM (corresponding to EGM-2 without FBS and supplements) for PCR experiments, or on gelatin-coated 24-well plates (Corning) in EGM for EIA analysis. After 2–4 days, cell medium was replaced by EBM in the absence or presence of EV-077 at different concentrations (10, 100, 300 or 600 nM). After 1 h of preincubation, either vehicle or TNFα (1 ng/ml) was added into the T25 flasks. Then, cells were stimulated with TNFα (1 ng/ml) for 6 h (still in the presence of EV-077).

2.4. SMC incubation

At 80% confluence, the SMCs were trypsinized and resuspended in either DMEM supplemented with 2% FCS (“low serum media”), or in SmGM2 kit medium containing 5% FCS, 2 ng/ml FGF, 0.5 ng/ml EGF, 5 μg/ml insulin (“growth factor media”). Subsequently, 5000 cells in 200 μL were added to each well in a 96-well plate and left to adhere overnight. Either vehicle or EV-077 (50 nM, 500 nM, 5 μM) was added to the wells 1 h before U46619 at different concentrations (0.1 nM–10 μM) and incubated for another 48 h. All experiments were repeated 3–5 times for each experimental condition.

2.5. Real time RT-PCR

After the incubation (*cf. supra*), cell medium was discarded and cells were washed twice with PBS. Total RNA extraction was performed by using QIAGEN RNeasy mini Kit according to the protocol provided by the manufacturer (QIAGEN). RNA concentration for each sample was determined by Nanodrop Spectrophotometer ND-100 (ISOGEN, Life Science). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit Roche (Roche Applied Science). Transcript levels were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). cDNA obtained by reverse transcription of total RNA were diluted 100 times and 5 μl were mixed with 12.5 μl of Fast start Universal SYBRGreen Master (Roche), 2.5 μl MilliQ water, 2.5 μl of diluted primer forward and 2.5 μl of diluted primer reverse to have an optimal and final concentration of 300 or 900 nM. PCRs were carried out in a real-time PCR cycler (Applied Biosystems 7900HT Fast Real-time PCR System): a hot start at 95 °C for 5 min was followed by 40 cycles at 95 °C for 15 s and 65 °C for 1 min. Samples were compared using the relative cycle threshold (C_t) method. To normalize the load of cDNA for each sample, RPL13 was used as the endogenous standard. Primer sequences are listed in Supplementary Table 1.

2.6. 6-Keto PGF1α enzyme immune assay

After the incubation (*cf. supra*), medium of each well was collected and cell lysis was performed in NaOH 0.5 N for 30 min. 6-Keto PGF1α levels in cell culture media were determined using a competitive assay (#515211, Cayman, USA). Results are expressed in picograms of 6-ketoPGF1α reported to micrograms of proteins measured by the Folin method (Folin–Ciocalteu’s phenol reagent, Merck, Germany) in cell lysates.

2.7. Measurements of SMC proliferation

SMC proliferation was evaluated using WST-1 reagent (Chemicon) according to the manufacturer’s instructions as previously described [27]. In brief, after replacement of phenol red containing media by 200 μL transparent serum-supplemented DMEM, 10 μL of diluted WST-1 reagent was added to each well at the end of the 48 h incubation period. After 1 h incubation at 37 °C, the absorbance of the formazan dye formed was measured at 440 nm using a microplate reader.

2.8. Data analysis

All results are expressed as mean ± SE. Statistically significant differences were determined by a one-way analysis of variances (ANOVA), followed by a Bonferroni *post hoc* test, for multiple comparisons. A p value of less than 0.05 was considered significant.

3. Results

3.1. Effect of EV-077 on HUVEC gene expression induced by TNFα

A statistically significant increase in mRNA expression of all genes of interest was observed upon TNFα stimulation, except for IL-6 for which the increase did not reach statistical significance (Fig. 1). For two of the studied genes of interest, ICAM-1 and PTX3, mRNA levels were significantly decreased in the presence of EV-077 from a concentration of 100 nM (10 nM had no effect). This effect was however not concentration-dependent, since inhibition was not increased at higher concentrations of EV-077 (300 and 600 nM; Fig. 1). EV-077 at the highest concentration (600 nM) did not affect mRNA expression in the absence of TNFα.

3.2. Effect of EV-077 and TNFα on HUVEC 6-keto PGF1α secretion

Although neither EV-077 nor TNFα alone increased 6-keto $PGF_{1\alpha}$ secretion, the simultaneous incubation of TNFα with EV-077 (600 nM) significantly increased 6-keto $PGF_{1\alpha}$ secretion (Fig. 2).

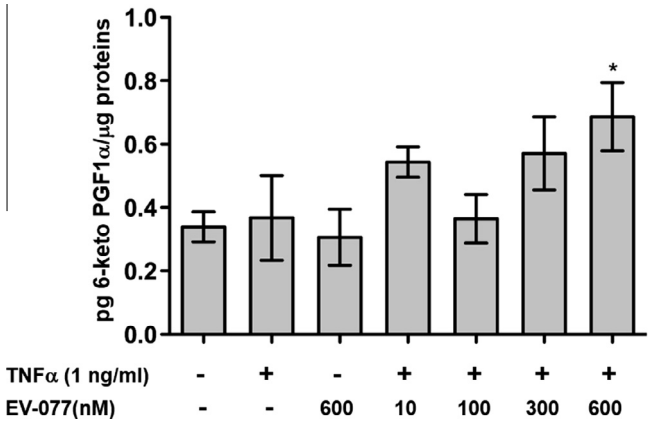

Fig. 2. Effects of EV-077 and TNFα on 6-keto PGF1α secretion by HUVEC after 6 h of incubation. 6-Keto PGF1α secretion was evaluated using a specific EIA assay. Results are expressed in picograms of 6-keto PGF1α reported to micrograms of proteins measured by the Folin method on cell lysates. Data are means ± S.D. ($n = 3$). Statistical analysis: one way ANOVA followed by Bonferroni, $^{*}P < 0.05$ vs. control.

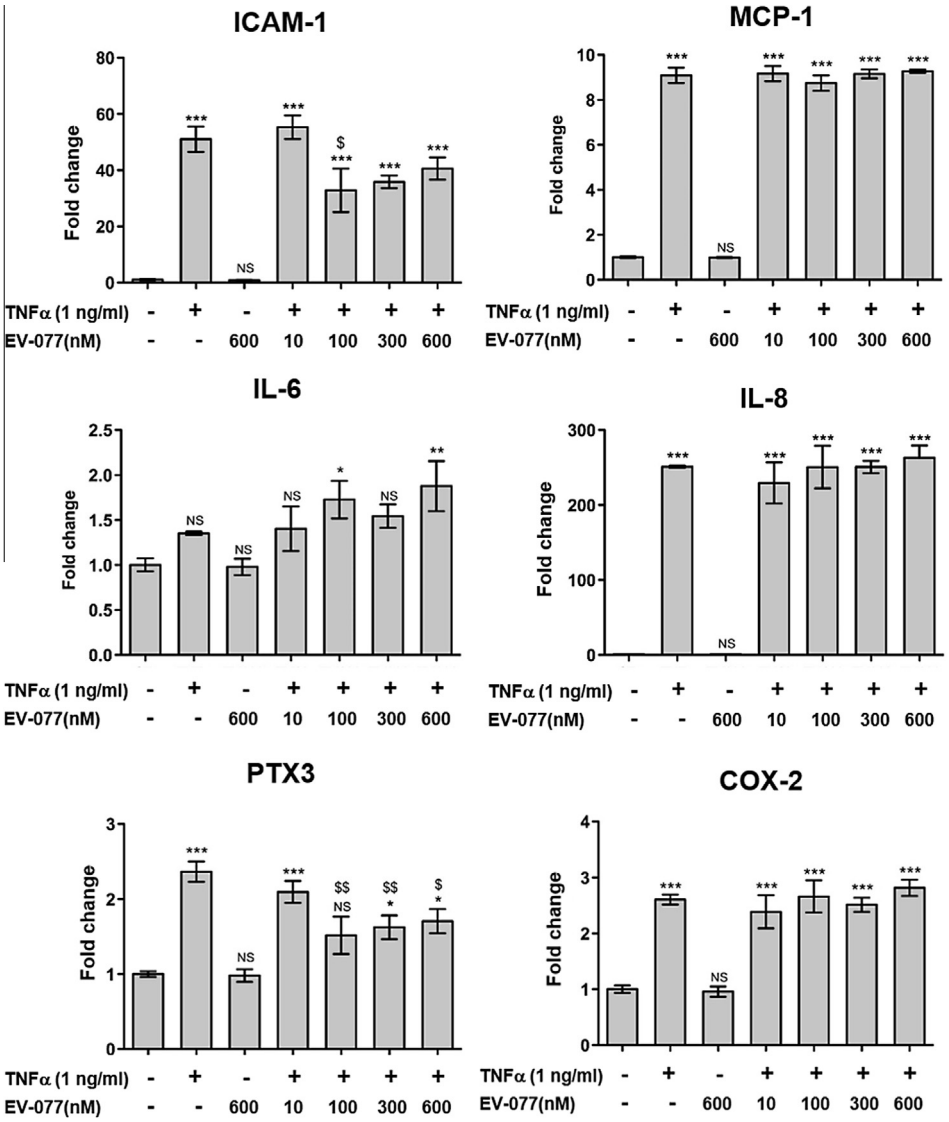

Fig. 1. Effects of EV-077 on mRNA expression of genes of interest encoding inflammatory or oxidative markers. mRNA expression was evaluated by quantitative RT-PCR in HUVEC after 6 h of TNFα stimulation. Data are means ± S.D. ($n = 3$). Statistical analysis: one way ANOVA followed by Bonferroni, versus control NS $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; versus TNFα $^{\$}p < 0.05$, $^{\$\$}p < 0.01$.

3.3. Effect of EV-077 and human coronary artery SMC proliferation

In the presence of growth factor media, the thromboxane mimetic U46619 induced a bell-shaped concentration-dependent increase in cell number as assessed by WST-1 (Fig. 3). The maximal response was observed at U46619-concentration of 1 nM, and at concentrations of 0.1 μM and higher, the absorbance did not significantly differ between U46619- and vehicle-treated cells (Fig. 3). In contrast to those findings, U46619 did not significantly alter SMC proliferation when experiments were performed in low serum media (Fig. 3).

In the presence of growth factor media, the dual TP receptor antagonist and thromboxane synthase inhibitor EV-077 significantly and concentration-dependently inhibited the SMC proliferation induced by U46619 (Fig. 4).

4. Discussion

The present study points to a role of thromboxane signaling through the TP receptor in cells that constitute the vascular wall, in terms of proinflammatory transcription in endothelial cells and vascular smooth muscle cell proliferation. These findings support a role of thromboxane/TP receptor pathway in alterations of the vascular wall associated with early atherosclerosis.

TNFα is a proinflammatory stimulus in HUVECs [28], and previous studies have shown that different TP receptor antagonists inhibit proinflammatory transcription induced by either TNFα or the thromboxane mimetic U46619 [29]. In the present study, TNFα-induced ICAM-1, MCP-1, IL-8, COX-2, and PTX3 but not IL-6 mRNA expression. Interestingly, EV-077 significantly decreased TNFα-induced ICAM-1 and PTX3 mRNA expression, whereas no significant effects on mRNA expression of the other genes of interest were observed. These results suggest that TP receptor signalling may be associated with a specific profile of transcriptional activation in HUVECs.

Whereas the signalling pathways involved in this response remains to be established, it should be taken into consideration that several mediators may be involved in the observed EV-077-induced effects on mRNA levels. In addition to TXA_2, the isoprostane 8-iso $PGF_{2\alpha}$, derived from free radical-induced oxidation of arachidonic acid, signal through the TP receptor, and EV-077 antagonizes also isoprostane-induced TP activation [23]. However, the EC activation by 8-iso $PGF_{2\alpha}$ may differ from that induced by TXA_2, as suggested by studies of leukocyte–EC interactions [30]. Furthermore, prostacyclin (PIG_2, Prostaglandin I_2) is formed from arachidonic acid mainly by the vascular endothelium and is a local potent vasodilator and inhibitor of platelet aggregation. PIG_2 is non-enzymatically hydrated to 6-keto $PGF_{1\alpha}$ in ECs. The present study revealed that HUVECs incubated with EV-077 released significantly higher amounts of 6-keto $PGF_{1\alpha}$ in response to TNFα, indicating an enhanced prostacyclin production which is known to exert anti-inflammatory effects in ECs [31].

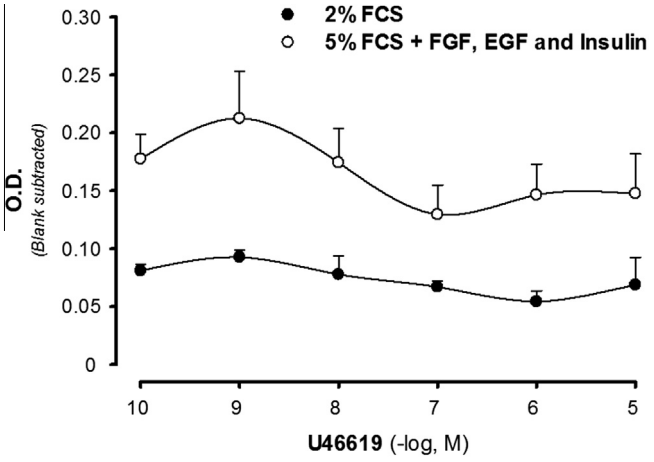

Fig. 3. Effect of the TP receptor agonist U46619 on the proliferation of human coronary artery smooth muscle cells, assessed by WST-1 reagent (see text). Experiments were performed either in the presence of FCS (2%), or in the presence of FCS (5%), FGF (2 ng/ml), EGF (0.5 ng/ml), and insulin (5 μg/ml). Results are expressed as mean ± SE for $n = 3$–7 observations at each concentration. All points were significantly different ($P < 0.05$) when comparing the two groups.

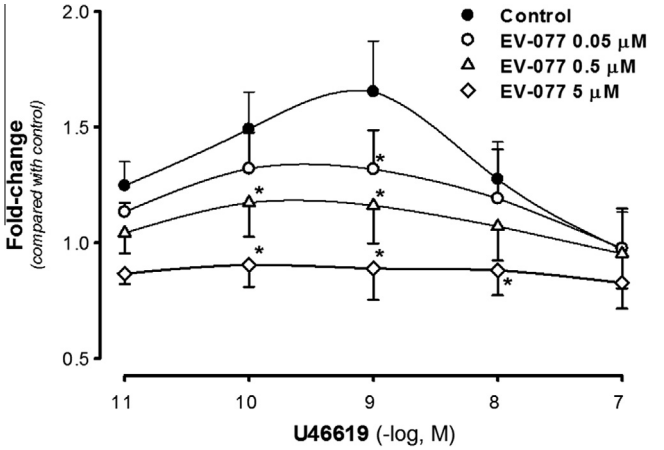

Fig. 4. Effect of the dual TP receptor antagonist and thromboxane synthase inhibitor EV-077 on the proliferation of human coronary artery smooth muscle cells induced by U46619, assessed by WST-1 reagent (see text). All experiments were performed in the presence of FCS (5%), FGF (2 ng/ml), EGF (0.5 ng/ml), and insulin (5 μg/ml). Results (mean ± SE) are expressed as fold change compared with vehicle treated cells (in the absence of U46619), $n = 7$. $^*P < 0.05$ vs. control at each concentration.

The increased cardiovascular risk associated with selective COX-2 inhibitors has been attributed to an imbalance between thromboxane and prostacyclin [9,32]. The present observations that EV-077 did not significantly alter the TNFα-induced COX-2 expression and increased 6-keto $PGF_{1\alpha}$ suggest that in addition to TP receptor antagonism and TXA synthase inhibition, this molecule may also restore the prostacyclin pathway in ECs. The notion of beneficial effects of the TP receptor antagonist EV-077 on endothelial function, which emerges from the present results, is in line with previous studies of terutroban [11,12]. However the clinical implications of these findings remain to be established, since the latter TP receptor antagonist was shown not be superior to aspirin in terms of cardiovascular outcome after 3.5 years follow-up in the PERFORM trial [33].

In the vascular wall, both ECs and vascular SMCs express TP receptors [9]. Whereas the thromboxane analogue U46619 did not alter SMC proliferation when cultured in low serum, a significant enhancement of proliferation was observed in the presence of growth factors and insulin in the present study. These results indicate that TP receptor signaling may not directly induce SMC proliferation, but rather acts as an amplification factor for vascular SMC proliferation in response to locally released mitogens. Previously, TXA_2 mimetics have been found to increase smooth muscle cell mitogenesis in the presence of a number of different growth stimuli, such as insulin [34], EGF, PDGF-BB [35,36], thrombin [37], oxidized LDL [38] or serum [15,18], supporting the notion of thromboxane as an enhancer of proliferation. However, in another study in which U46619 failed to stimulate bovine aortic SMC growth *per se*, this thromboxane mimetic did not significantly augment either PDGF- or EGF-induced proliferation [39], suggesting that the proliferation response transduced through the TP receptor may also depend on other factors.

In the present study, the tromboxane analogue U46619 induced a significant enhancement of SMC proliferation only at low concentrations. This concentration-dependent response is supported by previous studies for example in canine aortic SMC, which also exhibited a bell-shaped concentration–response proliferation curve [22]. However, in the latter study, maximal SMC proliferation, measured both by means of DNA synthesis and cell number, was observed at a U46619 concentration of 10 μM [22], which is substantially higher compared with the maximal effect observed at 1 nM of U46619 in the present study. Taken together, these findings suggest that human SMCs may be more sensitive to thromboxane stimulation compared with animal cells. However, methodological differences cannot be ruled out as the cause for these differential concentration–response curves.

The U46619 induced enhancement of SMC proliferation was significantly inhibited by EV-077 in the present study, supporting that low concentrations of U46619 induced an enhancement of SMC proliferation through TP receptor signaling and suggesting that TP receptor antagonism may reduce vascular hypertrophy associated with atherosclerosis. The latter notion has received support from animal models of hypertension and vascular injury in which either pharmacological or genetic targeting of the TP receptor in reduces vascular thickness [31,40]. Furthermore, increased urinary concentrations of 11-dehydro TXB_2 was recently demonstrated to be associated an increased intima media thickness of the carotid artery in subjects with obstructive sleep apnea [41].

The potent effects of the COX-pathway on the cardiovascular system have implications for several cardiovascular diseases such as atherosclerosis, restenosis, stroke, myocardial infarction [8,9,32], and more recently also demonstrated for atrial fibrillation [42]. In summary, the present study shows that the dual TP receptor antagonist and thromboxane synthase inhibitor EV-077 inhibited TNFα-induced endothelial inflammation and reduced the enhancement of SMC proliferation induced by a thromboxane mimetic. Taken together, these results provide support to the notion that TP receptor signaling may be associated with early atherosclerosis in terms of endothelial dysfunction and vascular hypertrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.078.

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